TITLE OF THE INVENTION

METHOD FOR PRODUCING L-GLUTAMIC ACID BY FERMENTATION ACCOMPANIED BY PRECIPITATION

BACKGROUND OF THE INVENTION

The present invention relates to a method for producing L-glutamic acid by fermentation accompanied by precipitation. L-Glutamic acid is widely used as a material for seasonings and so forth.

L-Glutamic acid is mainly produced by fermentative methods using so-called coryneform bacteria producing L-glutamic acid and belonging to the genus *Brevibacterium*, *Corynebacterium* or *Microbacterium*, or mutant strains thereof (Amino Acid Fermentation, pp.195-215, Gakkai Shuppan Center, 1986). As methods for producing L-glutamic acid by fermentation by using other bacterial strains, there are known a method using a microorganism belonging to the genus *Bacillus*, *Streptomyces*, *Penicillium* or the like (U.S. Patent No. 3,220,929), a method using a microorganism belonging to the genus *Pseudomonas*, *Arthrobacter*, *Serratia*, *Candida* or the like (U.S. Patent No. 3,563,857), a method using a microorganism belonging to the genus *Bacillus*, *Pseudomonas*, *Serratia*, *Aerobacter aerogenes* (currently referred to as *Enterobacter aerogenes*) or the like (Japanese Patent Publication (Kokoku) No. 32-9393), a method using a mutant strain of *Escherichia coli* (Japanese Patent Application Laid-open (Kokai) No. 5-244970) and so forth. In addition, the inventors of the present invention have proposed a method

for producing L-glutamic acid by using a microorganism belonging to the genus *Klebsiella, Erwinia* or *Pantoea* (Japanese Patent Application Laid-open No. 2000-106869).

Further, there have been disclosed various techniques for improving L-glutamic acid-producing ability by enhancing activities of L-glutamic acid biosynthetic enzymes through use of recombinant DNA techniques. For example, it has been reported that introduction of a gene coding for citrate synthase derived from *Escherichia coli* or *Corynebacterium glutamicum* was effective for enhancement of L-glutamic acid-producing ability in *Corynebacterium* or *Brevibacterium* bacteria (Japanese Patent Publication No. 7-121228). In addition, Japanese Patent Application Laid-open No. 61-268185 discloses a cell harboring recombinant DNA containing a glutamate dehydrogenase gene derived from *Corynebacterium* bacteria. Further, Japanese Patent Application Laid-open No. 63-214189 discloses a technique for improving L-glutamic acid-producing ability by amplifying a glutamate dehydrogenase gene, an isocitrate dehydrogenase gene, an aconitate hydratase gene and a citrate synthase gene.

Although L-glutamic acid productivity has been considerably increased by breeding of the aforementioned microorganisms or improvement of production methods, development of methods for more efficiently producing L-glutamic acid at a lower cost is required to respond to further increase of the demand in future.

There is known a method wherein fermentation is performed and crystallized L-amino acid is accumulated in

the culture (Japanese Patent Application Laid-open No. 62-288). In this method, the L-amino acid concentration in the culture is maintained below a certain level by precipitating the accumulated L-amino acid in the culture. Specifically, L-tryptophan, L-tyrosine or L-leucine is precipitated during fermentation by adjusting the temperature and pH of the culture or by adding a surface active agent to the medium.

While a fermentative method involving a precipitating L-amino acid is known as described above, amino acids suitable for this method are those of relatively low water solubility, and no example of applying the method to highly water-soluble amino acids such as L-glutamic acid is known. In addition, the medium must have low pH to precipitate L-glutamic acid. However, L-glutamic acid-producing bacteria such as those mentioned above cannot grow under acidic conditions, and therefore L-glutamic acid fermentation is performed under neutral conditions (U.S. Patent Nos. 3,220,929 and 3,032,474; Chao K.C. & Foster J.W., J. Bacteriol., 77, pp.715-725 (1959)). Thus, production of L-glutamic acid by fermentation accompanied by precipitation is not known. Furthermore, it is known that growth of most acidophile bacteria is inhibited by organic acids such as acetic acid, lactic acid and succinic acid (Yasuro Oshima Ed., "Extreme Environment Microorganism Handbook", p.231, Science Forum; Borichewski R.M., J. Bacteriol., 93, pp.597-599 (1967) etc.). Therefore, it is considered that many microorganisms are susceptible to L-glutamic acid, which is also an organic acid, under acidic

conditions, and there has been no report that search for microorganisms showing L-glutamic acid-producing ability under acidic conditions was attempted.

SUMMARY OF THE INVENTION

In the aforementioned current situation, an object of the present invention is to search for and breed a microorganism that produces L-glutamic acid under low pH conditions and to provide a method for producing L-glutamic acid using an obtained microorganism by fermentation with precipitating L-glutamic acid.

The inventors of the present invention considered during the study for improvement of L-glutamic acid productivity by fermentation that inhibition of the production by L-glutamic acid accumulated in a medium at a high concentration was one of the obstructions to the improvement of productivity. For example, cells have an excretory system and an uptake system for L-glutamic acid. However, if L-glutamic acid once excreted into the medium is incorporated into cells again, not only does the production efficiency fall, but also the L-glutamic acid biosynthetic reactions are inhibited as a result. In order to avoid the inhibition of production by such accumulation of L-glutamic acid at high concentration, the inventors of the present invention screened microorganisms that can proliferate under acidic conditions and in the presence of a high concentration of L-glutamic acid. As a result, they successfully isolated microorganisms having such properties from a soil, and thus

accomplished the present invention.

Thus, the present invention provides the following.

(1) A microorganism which can metabolize a carbon source at a specific pH in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source, and has ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration in the liquid medium at the pH.

(2) The microorganism according to (1), which can grow in the liquid medium.

(3) The microorganism according to (1) or (2), wherein the pH is not more than 5.0.

(4) The microorganism according to any one of (1) to (3), which has at least one of the following characteristics:

(a) the microorganism is enhanced in activity of an enzyme that catalyzes a reaction for biosynthesis of L-glutamic acid; and

(b) the microorganism is decreased in or deficient in activity of an enzyme that catalyzes a reaction branching from a biosynthetic pathway of L-glutamic acid and producing a compound other than L-glutamic acid.

(5) The microorganism according to (4), wherein the enzyme that catalyzes the reaction for biosynthesis of L-glutamic acid is at least one selected from citrate synthase, phosphoenolpyruvate carboxylase and glutamate dehydrogenase.

(6) The microorganism according to (4) or (5), wherein the enzyme that catalyzes the reaction branching from the

biosynthetic pathway of L-glutamic acid and producing a compound other than L-glutamic acid is α-ketoglutarate dehydrogenase.

(7) The microorganism according to any one of (1) to (6), wherein the microorganism belongs to the genus *Enterobacter*.

(8) The microorganism according to (7), which is *Enterobacter agglomerans*.

(9) The microorganism according to (8), which has a mutation that causes less extracellular secretion of a viscous material compared with a wild strain when cultured in a medium containing a saccharide.

(10) A method for producing L-glutamic acid by fermentation, which comprises culturing a microorganism as defined in any one of (1) to (9) in a liquid medium of which pH is adjusted to a pH at which L-glutamic acid is precipitated, to produce and accumulate L-glutamic acid and precipitate L-glutamic acid in the medium.

(11) A method for screening a microorganism suitable for producing L-glutamic acid by fermentation with precipitating L-glutamic acid in a liquid medium, which comprises inoculating a sample containing microorganisms into an acidic medium containing L-glutamic acid at a saturation concentration and a carbon source, and selecting a strain that can metabolize the carbon source.

(12) The method according to (11), wherein a strain that can grow in the medium is selected as the strain that can metabolize the carbon source.

(13) The method according to (11) or (12), wherein a pH of

the medium is not more than 5.0.

According to the method of the present invention, L-glutamic acid can be produced by fermentation with precipitating L-glutamic acid. As a result, L-glutamic acid in the medium is maintained below a certain concentration, and L-glutamic acid can be produced without suffering from the product inhibition by L-glutamic acid at a high concentration.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 shows a restriction map of a DNA fragment derived from *Enterobacter agglomerans* pTWVEK101.

Fig. 2 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the *sucA* gene derived from *Enterobacter agglomerans* and that derived from *Escherichia coli*. Upper sequence: *Enterobacter agglomerans*, lower sequence: *Escherichia coli* (the same shall apply hereafter).

Fig. 3 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the *sucB* gene derived from *Enterobacter agglomerans* and that derived from *Escherichia coli*.

Fig. 4 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the *sdhB* gene derived from *Enterobacter agglomerans* and that derived from *Escherichia coli*.

Fig. 5 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the *sucC* gene derived

from *Enterobacter agglomerans* and that derived from *Escherichia coli*.

Fig. 6 shows construction of plasmid pMWCPG having a *gltA* gene, a *ppc* gene and a *gdhA* gene.

Fig. 7 shows construction of plasmid RSF-Tet having the replication origin of the broad host spectrum plasmid RSF1010 and a tetracycline resistance gene.

Fig. 8 shows construction of plasmid RSFCPG having the replication origin of the broad host spectrum plasmid RSF1010, a tetracycline resistance gene, a *gltA* gene, a *ppc* gene and a *gdhA* gene.

Fig. 9 shows construction of plasmid pSTVCB having a *gltA* gene.

DETAILED DESCRIPTION OF THE INVENTION

Hereafter, the present invention will be explained in detail.

The microorganism of the present invention is a microorganism that (1) can metabolize a carbon source at a specific pH in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source and (2) has ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration in the liquid medium at the pH.

The term "saturation concentration" means a concentration of L-glutamic acid dissolved in a liquid medium when the liquid medium is saturated with L-glutamic acid.

Hereafter, a method for screening a microorganism that

can metabolize a carbon source in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source at a specific pH will be described. A sample containing microorganisms is inoculated into a liquid medium containing L-glutamic acid at a saturation concentration and a carbon source at a specific pH, and a strain that can metabolize the carbon source is selected. The specific pH is not particularly limited, but is usually not more than about 5.0, preferably not more than about 4.5, more preferably not more than about 4.3. The microorganism of the present invention is used to produce L-glutamic acid by fermentation with precipitating L-glutamic acid. If the pH is too high, it becomes difficult to allow the microorganism to produce L-glutamic acid enough for precipitation. Therefore, pH is preferably in the aforementioned range.

If pH of an aqueous solution containing L-glutamic acid is lowered, the solubility of L-glutamic acid significantly falls around pKa of γ-carboxyl group (4.25, 25°C). The solubility becomes the lowest at the isoelectric point (pH 3.2) and L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated. While it depends on the medium composition, L-glutamic acid is usually dissolved in an amount of 10 to 20 g/L at pH 3.2, 30 to 40 g/L at pH 4.0 and 50 to 60 g/L at pH 4.7, at about 30°C. Usually pH does not need to be made below 3.0, because the L-glutamic acid precipitating effect plateaus when pH goes below a certain value. However, pH may be below 3.0.

In addition, the expression that a microorganism "can

metabolize the carbon source" means that it can proliferate or can consume the carbon source even though it cannot proliferate, that is, it indicates that it catabolizes carbon sources such as saccharides or organic acids. Specifically, for example, if a microorganism proliferates when cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28°C, 37°C or 50°C for 2 to 4 days, this microorganism can metabolize the carbon source in the medium. Further, for example, even if a microorganism does not proliferate when it is cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28°C, 37°C or 50°C for 2 to 4 days, the microorganism which consumes the carbon source in the medium is that can metabolize the carbon source in the medium.

The microorganism which can metabolize the carbon source includes a microorganism which can grow in the liquid medium.

The expression that a microorganism "can grow" means that it can proliferate or can produce L-glutamic acid even though it cannot proliferate. Specifically, for example, if a microorganism proliferates when cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0,

more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28°C, 37°C or 50°C for 2 to 4 days, this microorganism can grow in the medium. Further, for example, even if a microorganism does not proliferate when it is cultured in a liquid synthetic medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28°C, 37°C or 50°C for 2 to 4 days, the microorganism which increases the amount of L-glutamic acid in the medium is that can grow in the medium.

The selection described above may be repeated two or more times under the same conditions or with changing pH or the concentration of L-glutamic acid. An initial selection can be performed in a medium containing L-glutamic acid at a concentration lower than the saturation concentration, and thereafter a subsequent selection can be performed in a medium containing L-glutamic acid at a saturation concentration. Further, strains with favorable properties such as superior proliferation rate may be selected.

In addition to the property described above, the microorganism of the present invention has ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration of L-glutamic acid in a liquid medium. The pH of the aforementioned liquid medium is preferably the same as or close to that of the medium used for screening a microorganism having the aforementioned property (1). Usually, a microorganism becomes susceptible

to L-glutamic acid at a high concentration as pH becomes lower. Therefore, it is preferred that pH is not low from the viewpoint of resistance to L-glutamic acid, but low pH is preferred from the viewpoint of production of L-glutamic acid with precipitating it. To satisfy these conditions, pH may be in the range of 3 to 5, preferably 4 to 5, more preferably 4.0 to 4.7, still more preferably 4.0 to 4.5, particularly preferably 4.0 to 4.3.

As the microorganism of the present invention or breeding materials therefor, there can be mentioned, for example, microorganisms belonging to the genus *Enterobacter*, *Klebsiella*, *Serratia*, *Pantoea*, *Erwinia*, *Escherichia*, *Corynebacterium*, *Alicyclobacillus*, *Bacillus*, *Saccharomyces* or the like. Among these, microorganisms belonging to the genus *Enterobacter* are preferred. Hereafter, the microorganism of the present invention will be explained mainly for microorganisms belonging to the genus *Enterobacter*, but the present invention can be applied to microorganism belonging to other genera and not limited to the genus *Enterobacter*.

As microorganisms belonging to the *Enterobacter*, there can be specifically mentioned *Enterobacter agglomerans*, preferably the *Enterobacter agglomerans* AJ13355 strain. This strain was isolated from a soil in Iwata-shi, Shizuoka, Japan as a strain that can proliferate in a medium containing L-glutamic acid and a carbon source at low pH.

The physiological properties of AJ13355 are as follows:

(1) Gram staining: negative

(2) Behavior against oxygen: facultative anaerobic

(3) Catalase: positive

(4) Oxidase: negative

(5) Nitrate-reducing ability: negative

(6) Voges-Proskauer test: positive

(7) Methyl Red test: negative

(8) Urease: negative

(9) Indole production: positive

(10) Motility: motile

(11) H_2S production in TSI medium: weakly active

(12) β-galactosidase: positive

(13) Saccharide-assimilating property:

Arabinose: positive

Sucrose: positive

Lactose: positive

Xylose: positive

Sorbitol: positive

Inositol: positive

Trehalose: positive

Maltose: positive

Glucose: positive

Adonitol: negative

Raffinose: positive

Salicin: negative

Melibiose: positive

(14) Glycerol-assimilating property: positive

(15) Organic acid-assimilating property:

Citric acid: positive

Tartaric acid: negative

Gluconic acid: positive

Acetic acid: positive

Malonic acid: negative

(16) Arginine dehydratase: negative

(17) Ornithine decarboxylase: negative

(18) Lysine decarboxylase: negative

(19) Phenylalanine deaminase: negative

(20) Pigment formation: yellow

(21) Gelatin liquefaction ability: positive

(22) Growth pH: growth is possible at pH 4.0, good growth at pH 4.5 to 7

(23) Growth temperature: good growth at 25°C, good growth at 30°C, good growth at 37°C, growth is possible at 42°C, growth is not possible at 45°C

Based on these bacteriological properties, AJ13355 was determined as *Enterobacter agglomerans*.

The *Enterobacter agglomerans* AJ13355 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305-8566, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on February 19, 1998 and received an accession number of FERM P-16644. It was then transferred to an international deposition under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6614.

The microorganism of the present invention may be a

microorganism originally having L-glutamic acid-producing ability or one having L-glutamic acid-producing ability imparted or enhanced by breeding through use of mutation treatment, recombinant DNA techniques or the like.

L-Glutamic acid-producing ability can be imparted or enhanced by, for example, increasing activity of an enzyme that catalyzes a reaction for biosynthesis of L-glutamic acid. L-Glutamic acid-producing ability can also be enhanced by decreasing activity of an enzyme that catalyzes a reaction branching from the biosynthetic pathway of L-glutamic acid and producing a compound other than L-glutamic acid, or making the activity deficient.

As enzymes that catalyze a reaction for biosynthesis of L-glutamic acid, there can be mentioned glutamate dehydrogenase (hereafter, also referred to as "GDH"), glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase (hereafter, also referred to as "CS"), phosphoenolpyruvate carboxylase (hereafter, also referred to as "PEPC"), pyruvate dehydrogenase, pyruvate kinase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and so forth. Among these enzymes, one, two or three of CS, PEPC and GDH are preferred. Further, it is preferred that the activities of all the three enzymes, CS, PEPC and GDH, are enhanced in the microorganism of the present invention. In particular,

CS of *Brevibacterium lactofermentum* is preferred, because it does not suffer from inhibition by α-ketoglutaric acid, L-glutamic acid and NADH.

In order to enhance the activity of CS, PEPC or GDH, for example, a gene coding for CS, PEPC or GDH may be cloned on an appropriate plasmid and a host microorganism may be transformed with the obtained plasmid. The copy number of the gene coding for CS, PEPC or GDH (hereafter, abbreviated as "*gltA* gene", "*ppc* gene" and "*gdhA* gene", respectively) in the transformed strain cell increases, resulting in the increase of the activity of CS, PEPC or GDH.

The cloned *gltA* gene, *ppc* gene and *gdhA* gene are introduced into the aforementioned starting parent strain solely or in combination of arbitrary two or three kinds of them. When two or three kinds of the genes are introduced, two or three kinds of the genes may be cloned on one kind of plasmid and introduced into the host, or separately cloned on two or three kinds of plasmids that can coexist and introduced into the host.

Two or more kinds of genes coding for enzymes of the same kind, but derived from different microorganisms may be introduced into the same host.

The plasmids described above are not particularly limited so long as they are autonomously replicable in cells of a microorganism belonging to, for example, the genus *Enterobacter* or the like, but, for example, there can be mentioned pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pH SG398, RSF1010, pMW119, pMW118, pMW219, pMW218, pACYC177,

pACYC184 and so forth. Besides these, vectors of phage DNA can also be used.

Transformation can be performed by, for example, the method of D.M. Morrison (Methods in Enzymology, 68, 326 (1979)), the method wherein permeability of DNA is increased by treating recipient bacterium cells with calcium chloride (Mandel M. and Higa A., J. Mol. Biol., 53, 159 (1970)), ~~the~~ electroporation (Miller J.H., "A Short Course in Bacterial Genetics", Cold Spring Harbor Laboratory Press, U.S.A. 1992) or the like.

The activity of CS, PEPC or GDH can also be increased by allowing multiple copies of a *gltA* gene, a *ppc* gene or a *gdhA* gene to be present on chromosomal DNA of the aforementioned starting parent strain serving as a host. In order to introduce multiple copies of the *gltA* gene, the *ppc* gene or the *gdhA* gene on chromosomal DNA of a microorganism belonging to the genus *Enterobacter* or the like, a sequence of which multiple copies are present on the chromosomal DNA, such as repetitive DNA and inverted repeats present at termini of a transposable element, can be used. Alternatively, multiple copies of the genes can be introduced onto chromosomal DNA by utilizing transfer of a transposon containing the *gltA* gene, the *ppc* gene or the *gdhA* gene. As a result, the copy number of the *gltA* gene, the *ppc* gene or the *gdhA* gene in a transformed strain cell is increased, and thus the activity of CS, PEPC or GDH is increased.

As organisms to be a source of the *gltA* gene, the *ppc* gene or the *gdhA* gene of which copy number is increased, any organism can be used so long as it has activity of CS, PEPC

or GDH. Inter alia, bacteria, which are prokaryotes, for example, those belonging to the genus *Enterobacter, Klebsiella, Erwinia, Pantoea, Serratia, Escherichia, Corynebacterium, Brevibacterium* and *Bacillus* are preferred. As specific examples, there can be mentioned *Escherichia coli*, *Brevibacterium lactofermentum* and so forth. The *gltA* gene, the *ppc* gene and the *gdhA* gene can be obtained from chromosomal DNA of the microorganisms described above.

The *gltA* gene, the *ppc* gene and the *gdhA* gene can be obtained by using a mutant strain which is deficient in the activity of CS, PEPC or GDH to isolate a DNA fragment which complements the auxotrophy from chromosomal DNA of the aforementioned microorganisms. Since the nucleotide sequences of these genes of *Escherichia* and *Corynebacterium* bacteria have already been elucidated (Biochemistry, 22, pp.5243-5249 (1983); J. Biochem., 95, pp.909-916 (1984); Gene, 27, pp.193-199 (1984); Microbiology, 140, pp.1817-1828 (1994); Mol. Gen. Genet., 218, pp.330-339 (1989); Molecular Microbiology, 6, pp.317-326 (1992)), they can also be obtained by PCR utilizing primers synthesized based on each nucleotide sequence and chromosomal DNA as a template.

The activity of CS, PEPC or GDH can also be increased by enhancing the expression of the *gltA* gene, the *ppc* gene or the *gdhA* gene besides the aforementioned amplification of the genes. For example, the expression can be enhanced by replacing a promoter for the *gltA* gene, the *ppc* gene or the *gdhA* gene with other stronger promoters. For example, *lac* promoter, *trp* promoter, *trc* promoter, *tac* promoter, P_R

promoter and P_L promoter of the lamda phage and so forth are known as strong promoters. The *gltA* gene, the *ppc* gene and the *gdhA* gene of which promoter is replaced are cloned on a plasmid and introduced into the host microorganism, or introduced onto the chromosomal DNA of the host microorganism by using repetitive DNA, inverted repeats, transposon or the like.

The activity of CS, PEPC or GDH can also be enhanced by replacing the promoter of the *gltA* gene, the *ppc* gene or the *gdhA* gene on the chromosome with other stronger promoters (see WO 87/03006 and Japanese Patent Application Laid-open No. 61-268183), or inserting a strong promoter in the upstream of the coding sequence of each gene (see Gene, 29, pp.231-241 (1984)). Specifically, homologous recombination can be performed between DNA containing the *gltA* gene, the *ppc* gene or the *gdhA* gene of which promoter is replaced with a stronger one or a part thereof and the corresponding gene on the chromosome.

Examples of the enzyme which catalyze a reaction branching from the biosynthetic pathway of the L-glutamic acid and producing a compound other than L-glutamic acid include α-ketoglutarate dehydrogenase (hereafter, also referred to as "αKGDH"), isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and so forth. Among these enzymes, αKGDH is preferred.

In order to obtain decrease or deficiency of the activity of the aforementioned enzyme in a microorganism belonging to the genus *Enterobacter* or the like, mutation causing decrease or deficiency of the intracellular activity of the enzyme can be introduced into the gene of the aforementioned enzyme by a usual mutagenesis or genetic engineering method.

Examples of the mutagenesis method include, for example, methods utilizing irradiation with X-ray or ultraviolet ray, methods utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine, and so forth. The site where the mutation is introduced to the gene may be in a coding region coding for an enzyme protein, or a region for regulating expression such as a promoter.

Examples of the genetic engineering methods include, for example, methods utilizing gene recombination, transduction, cell fusion and so forth. For example, a drug resistance gene is inserted into a cloned target gene to prepare a gene that has lost its function (defective gene). Subsequently, this defective gene is introduced into a cell of a host microorganism, and the target gene on the chromosome is replaced with the aforementioned defective gene by utilizing homologous recombination (gene disruption).

Decrease or deficiency of intracellular activity of the target enzyme and the degree of decrease of the activity can be determined by measuring the enzyme activity of a cell extract or a purified fraction thereof obtained from a candidate strain and comparing with that of a wild strain. For example, the αKGDH activity can be measured by the method

of Reed et al. (Reed L.J. and Mukherjee B.B., Methods in Enzymology, 13, pp.55-61 (1969)).

Depending on the target enzyme, the target mutant strain can be selected based on the phenotype of the mutant strain. For example, a mutant strain which is deficient in the αKGDH activity or decreases in the αKGDH activity cannot proliferate or shows a markedly reduced proliferation rate in a minimal medium containing glucose or a minimal medium containing acetic acid or L-glutamic acid as an exclusive carbon source under aerobic conditions. However, normal proliferation is enabled even under the same condition by adding succinic acid or lysine, methionine and diaminopimelic acid to a minimal medium containing glucose. By utilizing these phenomena as indicators, mutant strains with decreased αKGDH activity or deficient in the activity can be selected.

A method for preparing the αKGDH gene deficient strain of *Brevibacterium lactofermentum* by utilizing homologous recombination is described in detail in WO 95/34672. Similar methods can be applied to the other microorganisms.

Further, techniques such as cloning of genes and cleavage and ligation of DNA, transformation and so forth are described in detail in Molecular Cloning, 2nd Edition, Cold Spring Harbor Press, 1989 and so forth.

As a specific example of a mutant strain deficient in αKGDH activity or with decreased αKGDH activity obtained as described above, there can be mentioned *Enterobacter agglomerans* AJ13356. *Enterobacter agglomerans* AJ13356 was deposited at the National Institute of Bioscience and

Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305-8566, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on February 19, 1998 and received an accession number of FERM P-16645. It was then transferred to an international deposition under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6615. The *Enterobacter agglomerans* AJ13356 is deficient in αKGDH activity as a result of disruption of the αKGDH-E1 subunit gene (*sucA*).

When *Enterobacter agglomerans*, an example of the microorganism used in the present invention, is cultured in a medium containing a saccharide, a viscous material is extracellularly secreted, resulting in low operation efficiency. Therefore, when *Enterobacter agglomerans* having such a property of secreting the viscous material is used, it is preferable to use a mutant strain that secretes less the viscous material compared with a wild strain. Examples of mutagenesis methods include, for example, methods utilizing irradiation with X ray or ultraviolet ray, method utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine and so forth. A mutant strain with decreased secretion of the viscous material can be selected by inoculating mutagenized bacterial cells in a medium containing a saccharide, for example, LB medium plate containing 5 g/L of glucose, culturing them with tilting the plate about 45 degrees and selecting a colony which does not show flowing down of liquid.

In the present invention, impartation or enhancement of L-glutamic acid-producing ability and impartation of other favorable properties such as mutation for less viscous material secretion described above can be carried out in an arbitrary order.

By culturing the microorganism of the present invention in a liquid medium of which pH is adjusted to a pH at which L-glutamic acid is precipitated, L-glutamic acid can be produced and accumulated with precipitating it in the medium. L-Glutamic acid can also be precipitated by starting the culture at a neutral pH and then ending it at a pH at which L-glutamic acid is precipitated.

The pH at which L-glutamic acid is precipitated means one at which L-glutamic acid is precipitated when the microorganism produces and accumulates L-glutamic acid.

As the aforementioned medium, a usual nutrient medium containing a carbon source, a nitrogen source, mineral salts and organic trace nutrients such as amino acids and vitamins as required can be used so long as pH is adjusted to a pH at which L-glutamic acid is precipitated. Either a synthetic medium or a natural medium can be used. The carbon source and the nitrogen source used in the medium can be any ones so long as they can be used by the cultured strain.

As the carbon source, saccharides such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate and molasses are used. In addition, organic acids such as acetic acid and citric acid may be used each alone or in combination with another carbon source.

As the nitrogen source, ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate and ammonium acetate, nitrates and so forth are used.

As the organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, those containing these substances such as peptone, casamino acid, yeast extract and soybean protein decomposition products are used. When an auxotrophic mutant strain that requires an amino acid and so forth for metabolization or growth is used, the required nutrient must be supplemented.

As mineral salts, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and so forth are used.

As for the culture method, aeration culture is usually performed with controlling the fermentation temperature to be 20 to 42°C and pH to be 3 to 5, preferably 4 to 5, more preferably 4 to 4.7, particularly preferably 4 to 4.5. Thus, after about 10 hours to 4 days of culture, a substantial amount of L-glutamic acid is accumulated in the culture. Accumulated L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated in the medium.

After completion of the culture, L-glutamic acid precipitated in the culture can be collected by centrifugation, filtration or the like. L-Glutamic acid dissolved in the medium can be collected according to known methods. For example, the L-glutamic acid can be isolated by concentrating the culture broth to crystallize it or isolated by ion exchange chromatography or the like. L-Glutamic acid precipitated

in the culture broth may be isolated together with L-glutamic acid that has been dissolved in the medium after it is crystallized.

According to the method of the present invention, L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated, and the concentration of L-glutamic acid dissolved in the medium is maintained at a constant level. Therefore, the influence of L-glutamic acid at a high concentration on microorganisms can be reduced. Accordingly, it becomes possible to breed a microorganism having further improved L-glutamic acid-producing ability. Further, since L-glutamic acid is precipitated as crystals, acidification of the culture broth by accumulation of L-glutamic acid is suppressed, and therefore the amount of alkali used for maintaining pH of the culture can significantly be reduced.

EXAMPLES

Hereafter, the present invention will be more specifically explained with reference to the following examples.

<1> Screening of microorganism having L-glutamic acid resistance in acidic environment

Screening of a microorganism having L-glutamic acid resistance in an acidic environment was performed as follows. Each of about 500 samples obtained from nature including soil, fruits, plant bodies, river water in an amount of 1 g was suspended in 5 mL of sterilized water, of which 200 μL was

coated on 20 mL of solid medium of which pH was adjusted to 4.0 with HCl. The composition of the medium was as follows: 3 g/L of glucose, 1 g/L of $(NH_4)_2SO_4$, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of KH_2PO_4, 0.2 g/L of NaCl, 0.1 g/L of $CaCl_2 \cdot 7H_2O$, 0.01 g/L of $FeSO_4 \cdot 7H_2O$, 0.01 g/L of $MnSO_4 \cdot 4H_2O$, 0.72 mg/L of $ZnSO_4 \cdot 2H_2O$, 0.64 mg/L of $CuSO_4 \cdot 5H_2O$, 0.72 mg/L of $CoCl_2 \cdot 6H_2O$, 0.4 mg/L of boric acid, 1.2 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 50 μg/L of biotin, 50 μg/L of calcium pantothenate, 50 μg/L of folic acid, 50 μg/L of inositol, 50 μg/L of niacin, 50 μg/L of p-aminobenzoic acid, 50 μg/L of pyridoxine hydrochloride, 50 μg/L of riboflavin, 50 μg/L of thiamine hydrochloride, 50 mg/L of cycloheximide, 20 g/L of agar.

The media plated on which the above samples were plated were incubated at 28°C, 37°C or 50°C for 2 to 4 days and 378 strains each forming a colony were obtained.

Subsequently, each of the strains obtained as described above was inoculated in a test tube of 16.5 cm in length and 14 mm in diameter containing 3 mL of liquid medium (adjusted to pH 4.0 with HCl) containing a saturation concentration of L-glutamic acid and cultured at 28°C, 37°C or 50°C for 24 hours to 3 days with shaking. Then, the grown strains were selected. The composition of the aforementioned medium was follows: 40 g/L of glucose, 20 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 2 g/L of KH_2PO_4, 0.5 g/L of NaCl, 0.25 g/L of $CaCl_2 \cdot 7H_2O$, 0.02 g/L of $FeSO_4 \cdot 7H_2O$, 0.02 g/L of $MnSO_4 \cdot 4H_2O$, 0.72 mg/L of $ZnSO_4 \cdot 2H_2O$, 0.64 mg/L of $CuSO_4 \cdot 5H_2O$, 0.72 mg/L of $CoCl_2 \cdot 6H_2O$, 0.4 mg/L of boric acid, 1.2 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 2 g/L of yeast extract.

Thus, 78 strains of microorganisms having L-glutamic acid resistance in an acidic environment were successfully obtained.

<2> Selection of strains with superior growth rate in acidic environment from microorganisms having L-glutamic acid resistance

The various microorganisms having L-glutamic acid resistance in an acidic environment obtained as described above were each inoculated into a test tube of 16.5 cm in length and 14 mm in diameter containing 3 mL of medium (adjusted to pH 4.0 with HCl) obtained by adding 20 g/L of glutamic acid and 2 g/L of glucose to M9 medium (Sambrook, J., Fritsh, E.F. and Maniatis, T., "Molecular Cloning", Cold Spring Harbor Laboratory Press, 1989), and the turbidity of the medium was measured in the time course to select strains with a favorable growth rate. As a result, as a strain showing favorable growth, the AJ13355 strain was obtained from a soil in Iwata-shi, Shizuoka, Japan. This strain was determined as *Enterobacter agglomerans* based on its bacteriological properties described above.

<3> Acquisition of strain with less viscous material secretion from *Enterobacter agglomerans* AJ13355 strain

Since the *Enterobacter agglomerans* AJ13355 strain extracellularly secretes a viscous material when cultured in a medium containing a saccharide, operation efficiency is not favorable. Therefore, a strain with less viscous

material secretion was obtained by the ultraviolet irradiation method (Miller, J.H. et al., "A Short Course in Bacterial Genetics; Laboratory Manual", p.150, Cold Spring Harbor Laboratory Press, 1992).

The *Enterobacter agglomerans* AJ13355 strain was irradiated with ultraviolet ray for 2 minutes at the position 60 cm away from a 60-W ultraviolet lamp and cultured in LB medium overnight to fix mutation. The mutagenized strain was diluted and inoculated in LB medium containing 5 g/L of glucose and 20 g/L of agar so that about 100 colonies per plate would emerge and cultured at 30°C overnight with tilting the plate about 45 degrees, and then 20 colonies showing no flowing down of the viscous material were selected.

As a strain satisfying conditions that no revertant emerged even after 5 times of subculture in LB medium containing 5 g/L of glucose and 20 g/L of agar, and that there should be observed growth equivalent to the parent strain in LB medium, LB medium containing 5 g/L of glucose and M9 medium (Sambrook, J. et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Press, 1989) to which 20 g/L of L-glutamic acid and 2 g/L of glucose were added and of which pH was adjusted to 4.5 with HCl, SC17 strain was selected from the strains selected above.

<4> Construction of glutamic acid-producing bacterium from *Enterobacter agglomerans* SC17 strain

(1) Preparation of αKGDH deficient strain from *Enterobacter agglomerans* SC17 strain

A strain deficient in αKGDH and with enhanced L-glutamic acid biosynthetic system was prepared from the *Enterobacter agglomerans* SC17 strain.

(i) Cloning of αKGDH gene (hereafter, referred to as "*sucAB*") of *Enterobacter agglomerans* AJ13355 strain

The *sucAB* gene of the *Enterobacter agglomerans* AJ13355 strain was cloned by selecting a DNA fragment complementing the acetic acid-unassimilating property of the αKGDH-E1 subunit gene (hereafter, referred to as "*sucA*") deficient strain of *Escherichia coli* from chromosomal DNA of the *Enterobacter agglomerans* AJ13355 strain.

The chromosomal DNA of the *Enterobacter agglomerans* AJ13355 strain was isolated by a method usually employed when chromosomal DNA is extracted from *Escherichia coli* (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, pp.97-98, Baifukan, 1992). The pTWV228 (resistant to ampicillin) used as a vector was commercially available from Takara Shuzo Co., Ltd.

The chromosomal DNA of the AJ13355 strain digested with *Eco*T221 and pTWV228 digested with *Pst*I were ligated by using T4 ligase and used to transform the *sucA* deficient *Escherichia coli* JRG465 strain (Herbert, J. et al., Mol. Gen. Genetics, 105, 182 (1969)). A strain growing in an acetate minimal medium was selected from the transformant strains obtained above, and a plasmid was extracted from it and designated as pTWVEK101. The *Escherichia coli* JRG465 strain harboring pTWVEK101 recovered auxotrophy for succinic acid or L-lysine and L-methionine besides the acetic acid-assimilating

property. This suggests that pTWVEK101 contains the *sucA* gene of *Enterobacter agglomerans*.

Fig. 1 shows the restriction map of a DNA fragment derived from *Enterobacter agglomerans* in pTWVEK101. The determined nucleotide sequence of the hatched portion in Fig. 1 is shown as SEQ ID NO: 1. In this sequence, nucleotide sequences considered to be two full length ORFs and two nucleotide sequences considered to be partial sequences of the ORFs were found. SEQ ID NOS: 2 to 5 show amino acid sequences that can be encoded by these ORFs or partial sequences in an order from the 5′ end. As a result of a homology search for these, it was revealed that the portion of which nucleotide sequences were determined contained a 3′-end partial sequence of the succinate dehydrogenase iron-sulfur protein gene (*sdhB*), full length *sucA* and αKGDH-E2 subunit gene (*sucB*), and 5′-end partial sequence of the succinyl CoA synthetase β subunit gene (*sucC*). The results of a comparison of the amino acid sequences deduced from these nucleotide sequences with those derived from *Escherichia coli* (Eur. J. Biochem., 141, pp.351-359 (1984); Eur. J. Biochem., 141, pp.361-374 (1984); Biochemistry, 24, pp.6245-6252 (1985)) are shown in Figs. 2 to 5. Thus, the amino acid sequences each showed very high homology. In addition, it was found that a cluster of *sdhB-sucA-sucB-sucC* was constituted on the chromosome of *Enterobacter agglomerans* as in *Escherichia coli* (Eur. J. Biochem., 141, pp.351-359 (1984); Eur. J. Biochem., 141, pp.361-374 (1984); Biochemistry, 24, pp.6245-6252 (1985)).

(ii) Acquisition of αKGDH deficient strain derived from *Enterobacter agglomerans* SC17 strain

The homologous recombination was performed by using the *sucAB* gene of *Enterobacter agglomerans* obtained as described above to obtain an αKGDH deficient strain of *Enterobacter agglomerans*.

After pTWVEK101 was digested with *Sph*I to excise a fragment containing *sucA*, the fragment was blunt-ended with Klenow fragment (Takara Shuzo Co., Ltd.) and ligated with pBR322 digested with *Eco*RI and blunt-ended with Klenow fragment, by using T4 DNA ligase (Takara Shuzo Co., Ltd.). The obtained plasmid was digested at the restriction enzyme *Bgl*II recognition site positioned substantially at the center of *sucA* by using this enzyme, blunt-ended with Klenow fragment, and then ligated again by using T4 DNA ligase. It was considered that the *sucA* gene did not function because a frameshift mutation was introduced into *sucA* of the plasmid newly constructed through the above procedure.

The plasmid constructed as described above was digested with a restriction enzyme *Apa*LI, and subjected to agarose gel electrophoresis to recover a DNA fragment containing *sucA* into which the frameshift mutation was introduced and a tetracycline resistance gene derived from pBR322. The recovered DNA fragment was ligated again by using T4 DNA ligase to construct a plasmid for disrupting the αKGDH gene.

The plasmid for disrupting the αKGDH gene obtained as described above was used to transform the *Enterobacter agglomerans* SC17 strain by electroporation (Miller, J.H.,

"A Short Course in Bacterial Genetics; Handbook", p.279, Cold Spring Harbor Laboratory Press, U.S.A., 1992), and a strain wherein *sucA* on the chromosome was replaced with a mutant type one by homologous recombination of the plasmid was obtained by using the tetracycline resistance as an indicator. The obtained strain was designated as SC17sucA strain.

In order to confirm that the SC17sucA strain was deficient in the αKGDH activity, the enzyme activity was measured by the method of Reed et al. (Reed, L.J. and Mukherjee, B.B., Methods in Enzymology, 13, pp.55-61, (1969)) by using cells of the strain cultured in LB medium until the logarithmic growth phase. As a result, αKGDH activity of 0.073 (ΔABS/min/mg protein) was detected from the SC17 strain, whereas no αKGDH activity was detected from the SC17sucA strain, and thus it was confirmed that the *sucA* was deficient as proposed.

(2) Enhancement of L-glutamic acid biosynthetic system of *Enterobacter agglomerans* SC17sucA strain

Subsequently, a citrate synthase gene, a phosphoenolpyruvate carboxylase gene and a glutamate dehydrogenase gene derived from *Escherichia coli* were introduced into the SC17sucA strain.

(i) Preparation of plasmid having *gltA* gene, *ppc* gene and *gdhA* gene derived from *Escherichia coli*

The procedures of preparing a plasmid having a *gltA* gene, a *ppc* gene and a *gdhA* gene will be explained by referring

to Figs. 6 and 7.

A plasmid having a *gdhA* gene derived from *Escherichia coli,* pBRGDH (Japanese Patent Application Laid-open No. 7-203980), was digested with *Hin*dIII and *Sph*I, the both ends were blunt-ended by the T4 DNA polymerase treatment, and then the DNA fragment having the *gdhA* gene was purified and recovered. Separately, a plasmid having a *gltA* gene and a *ppc* gene derived from *Escherichia coli,* pMWCP (WO 97/08294), was digested with *Xba*I, and then the both ends were blunt-ended by using T4 DNA polymerase. This was mixed with the above purified DNA fragment having the *gdhA* gene and ligated by using T4 ligase to obtain a plasmid pMWCPG, which corresponded to pMWCP further containing the *gdhA* gene (Fig. 6).

At the same time, the plasmid pVIC40 (Japanese Patent Application Laid-open No. 8-047397) having the replication origin of the broad host spectrum plasmid RSF1010 was digested with *Not*I, treated with T4 DNA polymerase and digested with *Pst*I. pBR322 was digested with *Eco*T14I, treated with T4 DNA polymerase and digested with *Pst*I. The both products were mixed and ligated by using T4 ligase to obtain a plasmid RSF-Tet having the replication origin of RSF1010 and a tetracycline resistance gene (Fig. 7).

Subsequently, pMWCPG was digested with *Eco*RI and *Pst*I, and a DNA fragment having the *gltA* gene, the *ppc* gene and the *gdhA* gene was purified and recovered. RSF-Tet was similarly digested with *Eco*RI and *Pst*I, and a DNA fragment having the replication origin of RSF1010 was purified and recovered. The both products were mixed and ligated by using

T4 ligase to obtain a plasmid RSFCPG, which corresponded to RSF-Tet containing the *gltA* gene, the *ppc* gene and the *gdhA* gene (Fig. 8). It was confirmed that the obtained plasmid RSFCPG expressed the *gltA* gene, the *ppc* gene and the *gdhA* gene, by the complementation of the auxotrophy of the *gltA*, *ppc* or *gdhA* gene deficient strain derived from *Escherichia coli* and measurement of each enzyme activity.

(ii) Preparation of plasmid having *gltA* gene derived from *Brevibacterium lactofermentum*

A plasmid having the *gltA* gene derived from *Brevibacterium lactofermentum* was constructed as follows. PCR was performed by using the primer DNAs having the nucleotide sequences represented by SEQ ID NOS: 6 and 7, which were prepared based on the nucleotide sequence of the *Corynebacterium glutamicum gltA* gene (Microbiology, 140, pp.1817-1828 (1994)), and chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as a template to obtain a *gltA* gene fragment of about 3 kb. This fragment inserted into a plasmid pHSG399 (purchased from Takara Shuzo Co., Ltd.) digested with *Sma*I to obtain a plasmid pHSGCB (Fig. 9). Subsequently, pHSGCB was digested with *Hin*dIII, and the excised *gltA* gene fragment of about 3 kb was inserted into a plasmid pSTV29 (purchased from Takara Shuzo Co., Ltd.) digested with *Hin*dIII to obtain a plasmid pSTVCB (Fig. 9). It was confirmed that the obtained plasmid pSTVCB expressed the *gltA* gene, by measuring the enzyme activity in the *Enterobacter agglomerans* AJ13355 strain.

(iii) Introduction of RSFCPG and pSTVCB into SC17sucA strain

The *Enterobacter agglomerans* SC17sucA strain was transformed with RSFCPG by electroporation to obtain a transformant SC17sucA/RSFCPG strain having tetracycline resistance. Further, the SC17sucA/RSFCPG strain was transformed with pSTVCB by electroporation to obtain a transformant SC17sucA/RSFCPG+pSTVCB strain having chloramphenicol resistance.

<4> Acquisition of strain with improved resistance to L-glutamic acid in low pH environment

A strain with improved resistance to L-glutamic acid at a high concentration in a low pH environment (hereafter, also referred to as "high-concentration Glu-resistant strain at low pH") was isolated from the *Enterobacter agglomerans* SC17sucA/RSFCPG+pSTVCB strain.

The SC17sucA/RSFCPG+pSTVCB strain was cultured overnight at 30°C in LBG medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, 5 g/L of glucose), and the cells washed with saline was appropriately diluted and plated on an M9-E medium (4 g/L of glucose, 17 g/L of $Na_2HPO_4 \cdot 12H_2O$, 3 g/L of KH_2PO_4, 0.5 g/L of NaCl, 1 g/L of NH_4Cl, 10 mM of $MgSO_4$, 10 μM of $CaCl_2$, 50 mg/L of L-lysine, 50 mg/L of L-methionine, 50 mg/L of DL-diaminopimelic acid, 25 mg/L of tetracycline, 25 mg/L of chloramphenicol, 30 g/L of L-glutamic acid, adjusted to pH 4.5 with aqueous ammonia) plate. The colony emerged after culture at 32°C for 2 days was obtained

as a high-concentration Glu-resistant strain at low pH.

For the obtained strain, growth level in M9-E liquid medium was measured and L-glutamic acid-producing ability was tested in a 50-ml volume large test tube containing 5 ml of L-glutamic acid production test medium (40 g/L of glucose, 20 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 2 g/L of KH_2PO_4, 0.5 g/L of NaCl, 0.25 g/L of $CaCl_2 \cdot 7H_2O$, 0.02 g/L of $FeSO_4 \cdot 7H_2O$, 0.02 g/L of $MnSO_4 \cdot 4H_2O$, 0.72 mg/L of $ZnSO_4 \cdot 2H_2O$, 0.64 mg/L of $CuSO_4 \cdot 5H_2O$, 0.72 mg/L of $CoCl_2 \cdot 6H_2O$, 0.4 mg/L of boric acid, 1.2 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride, 25 mg/L of chloramphenicol). A strain that exhibited the best growth level and the same L-glutamic acid producing ability as that of its parent strain, the SC17/RSFCPG+pSTVCB strain, was designated as *Enterobacter agglomerans* AJ13601. The AJ13601 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305-8566, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on August 18, 1999 and received an accession number of FERM P-17516. It was then transferred to an international depository under the provisions of Budapest Treaty on July 6, 2000 and received an accession number of FERM BP-7207.

<5> Culture of *Enterobacter agglomerans* AJ13601 strain for L-glutamic acid production (1)

The *Enterobacter agglomerans* AJ13601 strain was inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 20 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 2 g/L of KH_2PO_4, 0.5 g/L of NaCl, 0.25 g/L of $CaCl_2 \cdot 7H_2O$, 0.02 g/L of $FeSO_4 \cdot 7H_2O$, 0.02 g/L of $MnSO_4 \cdot 4H_2O$, 0.72 mg/L of $ZnSO_4 \cdot 2H_2O$, 0.64 mg/L of $CuSO_4 \cdot 5H_2O$, 0.72 mg/L of $CoCl_2 \cdot 6H_2O$, 0.4 mg/L of boric acid, 1.2 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol, and cultured at 34°C and pH 6.0 for 14 hours. The culture pH was controlled by introducing ammonia gas into the medium.

The culture obtained as described above was centrifuged at 5000 rpm for 10 minutes, and the collected cells were inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 5 g/L of $(NH_4)_2SO_4$, 1.5 g/L of $MgSO_4 \cdot 7H_2O$, 6 g/L of KH_2PO_4, 1.5 g/L of NaCl, 0.75 g/L of $CaCl_2 \cdot 7H_2O$, 0.06 g/L of $FeSO_4 \cdot 7H_2O$, 0.06 g/L of $MnSO_4 \cdot 4H_2O$, 2.16 mg/L of $ZnSO_4 \cdot 2H_2O$, 1.92 mg/L of $CuSO_4 \cdot 5H_2O$, 2.16 mg/L of $CoCl_2 \cdot 6H_2O$, 1.2 mg/L of boric acid, 3.6 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 6 g/L of yeast extract, 600 mg/L of L-lysine hydrochloride, 600 mg/L of L-methionine, 600 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol and cultured at 34°C and pH 4.5 to perform culture for L-glutamic acid production. The culture pH was controlled by introducing ammonia gas into the medium. As the initially added glucose was depleted, 600 g/L of glucose was continuously

added.

As a result of the culture for L-glutamic acid production performed for 50 hours as described above, a substantial amount of L-glutamic acid crystals were precipitated in the jar fermenter. Table 1 shows the concentration of L-glutamic acid dissolved in the culture broth at that time and the L-glutamic acid concentration measured by dissolving the crystals in 2 M potassium hydroxide. L-Glutamic acid crystals were collected from the culture by decantation after the culture was left stood.

Table 1

Concentration of L-glutamic acid dissolved in culture broth	51 g/L
Amount of L-glutamic acid precipitated as crystals	67 g/L
Concentration of L-glutamic acid measured by dissolving crystals	118 g/L

<6> Culture of *Enterobacter agglomerans* AJ13601 strain for L-glutamic acid production (2)

The following experiment was performed in order to confirm that the *Enterobacter agglomerans* AJ13601 strain still had L-glutamic acid-producing ability even under the condition that L-glutamic acid crystals were present.

The *Enterobacter agglomerans* AJ13601 strain was inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 20 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 2 g/L of KH_2PO_4, 0.5 g/L of NaCl, 0.25 g/L of $CaCl_2 \cdot 7H_2O$, 0.02 g/L of $FeSO_4 \cdot 7H_2O$, 0.02 g/L of $MnSO_4 \cdot 4H_2O$, 0.72

mg/L of $ZnSO_4 \cdot 2H_2O$, 0.64 mg/L of $CuSO_4 \cdot 5H_2O$, 0.72 mg/L of $CoCl_2 \cdot 6H_2O$, 0.4 mg/L of boric acid, 1.2 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol, and cultured at 34°C at pH 6.0 for 14 hours. The culture pH was controlled by bubbling the medium with ammonia gas. The culture obtained as described above was centrifuged at 5000 rpm for 10 minutes, and then the collected cells were cultured in a medium where L-glutamic acid was present as crystals. The used medium contained 40 g/L of glucose, 5 g/L of $(NH_4)_2SO_4$, 1.5 g/L of $MgSO_4 \cdot 7H_2O$, 6 g/L of KH_2PO_4, 1.5 g/L of NaCl, 0.75 g/L of $CaCl_2 \cdot 7H_2O$, 0.06 g/L of $FeSO_4 \cdot 7H_2O$, 0.06 g/L of $MnSO_4 \cdot 4H_2O$, 2.16 mg/L of $ZnSO_4 \cdot 2H_2O$, 1.92 mg/L of $CuSO_4 \cdot 5H_2O$, 2.16 mg/L of $CoCl_2 \cdot 6H_2O$, 1.2 mg/L of boric acid, 3.6 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 6 g/L of yeast extract, 600 mg/L of L-lysine hydrochloride, 600 mg/L of L-methionine, 600 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol and L-glutamic acid crystals were added to 40 g/L. The cells were inoculated in a 1-L jar fermenter containing 300 ml of this medium and cultured at 34°C and pH 4.3 to perform culture for L-glutamic acid production. The culture pH was controlled by introducing ammonia gas into the medium. As the initially added glucose was depleted, 600 g/L of glucose was continuously added. In this medium, only 39 g/L of the added L-glutamic acid was dissolved at pH 4.3 and the remaining 1 g/L was present as crystals.

As a result of the culture for L-glutamic acid production performed for 53 hours as described above, a substantial amount of L-glutamic acid crystals were precipitated in the jar fermenter. Table 2 shows the concentration of L-glutamic acid dissolved in the culture broth, the amount of L-glutamic acid present as crystals at that time and the L-glutamic acid concentration measured by dissolving the crystals in 2 M KOH solution. L-Glutamic acid crystals were collected from the culture by decantation after the culture was left stood. The results showed that the *Enterobacter agglomerans* AJ13601 strain accumulated L-glutamic acid and precipitated crystals thereof even under the condition that L-glutamic acid crystals were present.

Table 2

Concentration of L-glutamic acid dissolved in culture broth	39 g/L
Amount of L-glutamic acid precipitated as crystals	119 g/L
Concentration of L-glutamic acid measured by dissolving crystals	158 g/L
Amount of L-glutamic acid crystals newly produced by main culture	118 g/L

<7> Culture of *Enterobacter agglomerans* AJ13601 strain for L-glutamic acid production (3)

The *Enterobacter agglomerans* AJ13601 strain can grow not only at an acidic pH, but also at a neutral pH. Therefore, it was confirmed as follows that L-glutamic acid crystals could also be precipitated by starting the culture at a neutral pH and allowing production of L-glutamic acid during the

culture so that pH of the culture should spontaneously be lowered.

Cells of one plate (8.5 cm in diameter) of the *Enterobacter agglomerans* AJ13601 strain, cultured on LBG agar medium (10 g/ of L tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, 5 g/L of glucose, 15 g/L of agar) containing 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol at 30°C for 14 hours, were inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 5 g/L of $(NH_4)_2SO_4$, 1.5 g/L of $MgSO_4 \cdot 7H_2O$, 6 g/L of KH_2PO_4, 1.5 g/L of NaCl, 0.75 g/L of $CaCl_2 \cdot 7H_2O$, 0.06 g/L of $FeSO_4 \cdot 7H_2O$, 0.06 g/L of $MnSO_4 \cdot 4H_2O$, 2.16 mg/L of $ZnSO_4 \cdot 2H_2O$, 1.92 mg/L of $CuSO_4 \cdot 5H_2O$, 2.16 mg/L of $CoCl_2 \cdot 6H_2O$, 1.2 mg/L of boric acid, 3.6 mg/L of $Na_2MoO_4 \cdot 2H_2O$, 6 g/L of yeast extract, 600 mg/L of L-lysine hydrochloride, 600 mg/L of L-methionine, 600 mg/L of DL-α,ε-diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol and the culture was started at 34°C and pH 7.0. The culture pH was controlled by introducing ammonia gas into the medium. As the initially added glucose was depleted, 600 g/L of glucose was continuously added.

As L-glutamic acid is accumulated, the pH decreases spontaneously. The amount of the introduced ammonia gas was adjusted so that pH should be gradually lowered from 7.0 to 4.5 during the period between 15 hours and 24 hours after the start of the culture, and 24 hours after the start of the culture, pH became 4.5. Afterward, cultivation was continued for 12 hours.

As a result of the culture for L-glutamic acid production conducted for 36 hours as described above, a substantial amount of L-glutamic acid crystals were precipitated in the jar fermenter. Table 3 shows the concentration of L-glutamic acid dissolved in the culture broth, the amount of L-glutamic acid present as crystals at that time and the L-glutamic acid concentration measured by dissolving the crystals in 2 M KOH solution. L-Glutamic acid crystals were collected from the culture by decantation after the culture was left to stand.

Table 3

Concentration of L-glutamic acid dissolved in culture broth	45 g/L
Amount of L-glutamic acid precipitated as crystals	31 g/L
Concentration of L-glutamic acid measured by dissolving crystals	76 g/L